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Therapeutic angiogenesis in a murine model of limb ischemia by recombinant periostin and its fasciclin I domain

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ABSTRACT

Periostin, an extracellular matrix protein, is expressed in injured tissues, such as the heart with myocardial infarction, and promotes angiogenesis and tissue repair. However, the molecular mechanism associated with periostin-stimulated angiogenesis and tissue repair is still unclear. In order to clarify the role of periostin in neovascularization, we examined the effect of periostin in angiogenic potentials of human endothelial colony forming cells (ECFCs) *in vitro* and in an ischemic limb animal model. Recombinant periostin protein stimulated the migration and tube formation of ECFCs. To identify the functional domains of periostin implicated in angiogenesis, five fragments of periostin, including four repeating FAS-1 domains and a carboxyl terminal domain, were expressed in *Escherichia coli* and purified to homogeneity. Of the five different domains, the first FAS-1 domain stimulated the migration and tube formation of human ECFCs as potent as the whole periostin. Chemotactic migration of ECFCs induced by the full length and the first FAS-1 domain of periostin was abrogated by blocking antibodies against β3 and β5 integrins. Intramuscular injection of the full length and the first FAS-1 domain of periostin into the ischemic hindlimb of mice attenuated severe limb loss and promoted blood perfusion and homing of intravenously administered ECFCs to the ischemic limb. These results suggest that the first FAS-1 domain is responsible for periostin-induced migration and angiogenesis and it can be used as a therapeutic tool for treatment of peripheral artery occlusive disease by stimulating homing of ECFCs.

1. Introduction

Peripheral artery disease (PAD) is a common vascular disease that not only affects the quality of life but also increases the risk of cardiovascular events. PAD can develop in association with any disease causing stenosis or occlusion of the lower limb arteries; atherosclerosis disease is the most common etiology. PAD has been reported in diverse races in 3–10% of people aged younger than 70 years and in 15–20% of people older than 70 years. One in five patients older than 65 years had either symptomatic or asymptomatic peripheral arterial disease. One third of PAD patients tend to have a complete occlusion of a major artery to the leg as they age. Patients with diabetic peripheral arterial disease are at the highest risk of progression toward critical limb ischemia, have a 10 times greater possibility of requiring amputation, and have a prevalence of gangrene 20–30 times higher than that of the general population [1]. Therapeutic angiogenesis is important for blood perfusion in ischemic tissues and tissue repair after critical ischemia [2–5].

Endothelial colony forming cells (ECFCs) were first isolated from the adult peripheral blood in 1997 [6]. ECFCs were also identified in the bone marrow and other tissues, representing a highly pro-angiogenic pool of cells liable to accumulate into foci of physiological and pathological neovascularization [6,7]. ECFCs exhibit characteristics usually associated to common stem/progenitor cells. It is thought that ECFCs are able to maintain their immature state and, upon an encounter with appropriate stimuli, they are able to migrate, proliferate, or differentiate into endothelial cells. In addition, ECFCs can also contribute directly to regenerative processes or at least support the regeneration of the injured cardio-vascular system [8]. Prior studies have shown that utilization of ECFCs is an encouraging approach for therapeutic applications as they may release angiogenic cytokines and participate in vascular regeneration [9]. A number of clinical and experimental trials have demonstrated the safety and feasibility of ECFC-based therapy in animal models of ischemia, such as critical limb ischemia and cardiovascular

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; ECFCs, endothelial colony forming cells; LDPI, laser Doppler perfusion imaging; PAD, peripheral artery disease

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diseases [10,11]; however, therapeutic potential of ECFC transplantation is limited due to poor engraftment and homing ability of transplanted ECFCs into ischemic tissues [12]. To improve the therapeutic potential of ECFCs, it is necessary to improve homing and engraftment potentials of transplanted ECFCs.

Periostin, originally identified as an osteoblast-specific factor-2, is a 93-kDa extracellular matrix protein, which shares a homology with the insect cell adhesion molecule fasciclin I (FAS-1). High expression of periostin in the periodontal ligament and the periosteum has been reported [13]. Periostin is involved in the regulation of cell adhesion and motility through an integrin-dependent mechanism [14]. Periostin possesses an N-terminal EMI domain which promotes the formation of multimers in non-reducing conditions, a tandem repeat of four FAS-1 domains which act as ligands for integrins, and a C-terminal domain which regulates cell–matrix organization and interactions by binding extracellular matrix proteins [13]. Periostin was reported to bind the integrins αvβ3, αvβ5, and α6β4, thereby promoting activation of diverse signaling pathways, including phosphoinositide-3-kinase-Akt, MEK–MAPK, and Src [15]. The functions of periostin are associated with skeletal development, heart development, and cancer [16–18]. Periostin promoted angiogenesis, migration, and invasion of human umbilical endothelial cells [19] and stimulated tumor angiogenesis in breast cancer [20]. These results suggest the potential for application of periostin in therapeutic angiogenesis of PAD and regulation of angiogenic activities of ECFCs.

In this study, we attempted to determine whether periostin can enhance angiogenic activities of ECFCs *in vitro* and *in vivo*. We identified the first FAS-1 domain as the functional domain of periostin implicated in the periostin-stimulated angiogenic activities of ECFCs. In addition, we explored the effects of the first FAS-1 domain of periostin in the regeneration and neovascularization of the ischemic limb and *in vivo* homing ability of transplanted ECFCs.

2. Materials and methods

2.1. Materials

Fetal bovine serum, trypsin, and CellTracker™ CM-DiI (CM-DiI) were purchased from Invitrogen (Carlsbad, CA). Endothelial Growth Medium-2 bullet kit was purchased from Lonza (Basel, Swiss). Culture plates were purchased from Nunc (Roskilde, Denmark). A recombinant human periostin protein was purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-α-SMA antibody (ab5694) was purchased from Abcam PLC (Cambridge, UK). Anti-CD31 rat antibody (MEC 13.3) and growth factor-reduced Matrigel™ were purchased from BD Biosciences (Franklin Lakes, NJ). Peroxidase-labeled secondary antibodies and Enhanced Chemiluminescence Western blotting system were purchased from Amersham Biosciences (Piscataway, NJ).

2.2. Expression and purification of recombinant periostin proteins

For the expression of functional domains of periostin as recombinant His_6-tagged proteins, four FAS-1 and C-terminal domains of human periostin were subcloned into a pET-30a expression vector (Novagen, Madison, WI) by ligation of PCR-generated cDNA fragments containing additional EcoRI and Hind III sites into the pET-30a vector. The amino acid sequences of the five domains of periostin are as follows: the first FAS-1 (representing amino acids 94 to 234), the second FAS-1 (amino acids 232 to 368), the third FAS-1 (amino acids 364 to 496), the fourth FAS-1 (amino acids 491 to 634), and the C-terminal domain (amino acids 627 to 780). We named these five fragments "domains 1 to 5" as shown in Fig. 1A. The His-tagged recombinant proteins were expressed in BL21 (DE3) cells, and were harvested and purified using a nickel-NTA agarose column (Qiagen, Inc., Valencia, CA) as described in the instruction manual. Endotoxin was removed using Detoxi-Gel Endotoxin Removing Gel (Pierce, Rockford, IL), and the removal of endotoxin was confirmed by *Limulus* Amebocyte Lysate test (Cape cod, East Falmouth, MA). The purified recombinant proteins were loaded onto 15% sodium dodecyl sulfate-acrylamide gel for electrophoresis and stained with Coomassie Brilliant Blue staining.

2.3. Cell culture

Human ECFCs were isolated from the human umbilical cord blood, which was collected in disposable sterile pyrogen-free bags (Green Cross, Yongin, Korea) containing anticoagulant. Written informed consent was obtained from all donors and the study was approved by the Institutional Review Board of Pusan National University Hospital. Mononuclear cells were isolated from the blood with Histopaque-1077 (Sigma-Aldrich, Switzerland) as described previously [21]. Human umbilical vein endothelial cells were purchased from Lonza (Allendale, NJ). Both ECFCs and human umbilical vein endothelial cells were seeded on culture dishes coated with 0.1% gelatin (Sigma-Aldrich) and maintained in endothelial cell basal medium-2 (EBM-2) (Clonetics, San Diego, CA) supplemented with EGM-2 MV Single Quotes containing 5% fetal bovine serum (FBS), human VEGF-1, human fibroblast growth factor-2 (FGF-2), human epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and ascorbic acid. After four days in culture, non-adherent cells were removed and adherent cells were trypsinized and re-plated at a density of 1×10^6 per well until day 7 [22].

2.4. Cell migration assay

Assay for the migration of ECFCs was performed using a disposable 96-well chemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD). Briefly, ECFCs were harvested with 0.05% trypsin containing 0.02% EDTA, washed once, and suspended in EBM-2 at a concentration of 1×10^5 cells/ml. A membrane filter with 8-μm pores for the chemotaxis chamber was pre-coated overnight with 20 μg/ml rat-tail collagen at 4 °C; an aliquot (50 μl) of ECFC suspension was loaded into the upper chamber, and EBM-2 supplemented with recombinant proteins of periostin was then placed in the lower chamber. Following incubation of the cells for 12 h at 37 °C, the filters were disassembled, and the upper surface of each filter was scraped free of cells by wiping it with a cotton swab. The number of cells that had migrated to the lower surface of each filter was determined by counting the cells in four locations under microscopy at 100× magnification after staining with Hoechst.

To clarify the involvement of integrins in the periostin-stimulated migration of ECFCs, cells were pre-incubated with function-blocking monoclonal antibodies specific to different types of integrins, such as integrins β_1 (HMβ1-1, BioLegend), β_3 (F11, BD), and β_5 (P1F6, Millipore), or control antibody (each 5 μg/ml) at 37 °C for 30 min. The antibody-incubated cells were then loaded into the upper chamber of a 96-well chemotaxis chamber pre-coated with rat-tail collagen, and migration of ECFCs was determined after incubation with recombinant periostin proteins for 12 h.

2.5. Cell adhesion assay

Ninety-six-well microculture plates (Falcon, Becton-Dickinson, Mountain View, CA) were incubated with recombinant periostin proteins or recombinant periostin domains 1 to 5 at 37 °C for 1 h, followed by blocking with PBS containing 0.2% BSA for 1 h at 37 °C. Cells were trypsinized and suspended in the culture media at a density of 2×10^5 cells/ml, and 0.1 ml of the cell suspension was then added to each well of the plate. Analysis of cell attachment was performed as follows. After incubation for 1 h at 37 °C, unattached cells were removed by rinsing twice with PBS. The number of attached cells was determined by counting the cells under microscopy at 100× magnification after staining with hematoxylin and eosin.

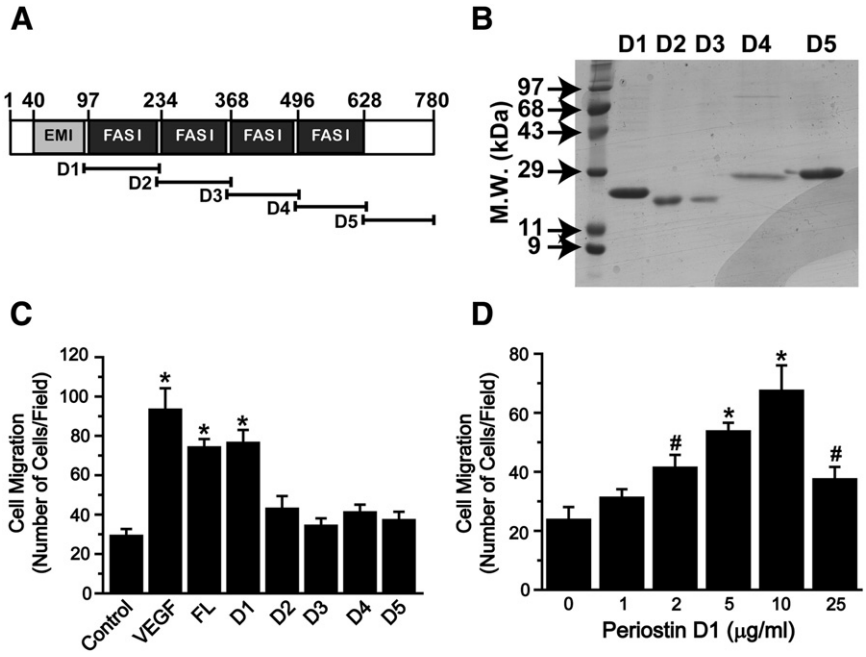

Fig. 1. Periostin stimulates the migration of ECFCs through the first FAS-1 domain. (A) Schematic diagram of human periostin and its five truncated protein constructs (D1, D2, D3, D4, and D5). (B) SDS-PAGE and Coomassie Brilliant Blue staining of the five periostin fragments. (C) Effects of the full length (FL) and the five fragments of periostin on migration of ECFCs. Chemotactic migration of ECFCs was determined after treatment with VEGF (10 ng/ml), full length recombinant periostin (10 μg/ml), or five fragments of periostin (each 10 μg/ml). (D) Dose-dependent effect of the first FAS-1 domain of periostin (periostin D1) on chemotactic migration of ECFCs. Migration of ECFCs in response to increasing concentrations of periostin D1 fragment was quantified. Data represent mean ± S.D. (n = 5). *, $p < 0.01$; #, $p < 0.05$ *vs* control.

2.6. Tube formation assay

Human ECFCs were maintained in a 37 °C, 5% CO_2 humidified environmental chamber. The cells were grown in an Endothelial Growth Medium-2 bullet kit (Lonza) according to the manufacturer's instructions. For tube formation assay of ECFCs, aliquots (50 μl) of growth factor-reduced Matrigel (10 mg protein/ml) were added to 96-well culture dishes and polymerized for 30 min at 37 °C. ECFCs were trypsinized, resuspended in EBM-2 basal medium supplanted with 1% FBS, and plated onto a layer of Matrigel at a density of 1×10^5 cells/well. The cells were then exposed to EBM-2 media with 1% FBS or recombinant periostin domains 1 to 5. After incubation of the Matrigel cultures at 37 °C for 9 h, the cultures were photographed using an inverted microscope with a digital camera.

2.7. Hindlimb ischemia, cell transplantation, and blood flow measurement

Animal experiments were performed using protocols approved by the Pusan National University Institutional Animal Use and Care Committee. C57BL/6J mice (male, age 8–10 wks, weighing 22–24 g) were anesthetized with an intraperitoneal injection of 400 mg/kg 2,2,2-tribromoethanol (Avertin; Sigma) for operative resection of one femoral artery and laser Doppler perfusion imaging. To determine the effects of the full length or the first FAS-1 domain of periostin on *in vivo* blood perfusion and tissue necrosis in ischemic limbs, eight different mice per each experimental group were injected with the recombinant proteins. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. After arterial ligation, ischemic hindlimbs were injected with the full length or fragments of periostin into three sites (20 μl/each site) of the gracilis muscle in the medial thigh three times per week. The extent of necrosis in the ischemic hindlimb was recorded on day 28 after surgery. Scores for necrosis were assessed as follows: 0, limb salvage; 1, toes amputation; 2, foot amputation; and 3, limb amputation. Blood flow of the ischemic and normal limbs was measured using a laser Doppler perfusion imaging (LDPI) analyzer (Moor instruments, Devon, UK) on days 0, 7, 14, 21, and 28 after induction of hindlimb ischemia. Perfusion of the ischemic and non-ischemic limbs was calculated on the basis of colored histogram pixels. Red and blue colors indicate high and low perfusion, respectively. Blood perfusion is expressed as the LDPI index representing the ratio of the ischemic *versus* non-ischemic limb blood flows. A ratio of 1 before surgery indicates equal blood perfusion of both legs.

2.8. Histological and immunofluorescence analyses

For histological and immunostaining of the tissue specimens, hindlimb muscles were removed, formalin-fixed, and paraffin-embedded. Three 6 μm-thick sections were taken from the paraffin-embedded specimens at 150 μm intervals, stained with hematoxylin and eosin (H&E), observed, and photographed with a microscope (Axioimager M2, Carl Zeiss, Heidenheim, Germany). Masson's trichrome collagen staining was also performed for the assessment of tissue fibrosis in ischemic regions. Endothelial cells and smooth muscle cells were immunostained with rabbit anti-CD31 and rabbit anti-α-SMA antibodies. Inflammatory cells were stained with rat anti-CD68 antibody (AbD Serotec, Raleigh, NC). The specimens were incubated with Alexa 488 goat anti-rat or Alexa 568 goat anti-rabbit secondary antibodies (Life Technologies, Carlsbad, CA), followed by washing and mounting in Vectashield medium (Vector Laboratories) with 4′,6-diamidino-2-phenylindole (DAPI) for visualization of nuclei. The stained sections were visualized by laser scanning confocal microscopy (Olympus FluoView FV1000). Capillary density, the number of arterioles/arteries, and the number of proliferating cells were assessed by counting the number of CD31-positive and α-SMA-positive features per high power field (400×). Twelve randomly chosen microscopic

fields from three serial sections in each tissue block were examined for the number of capillary density and α-SMA-positive arteries for each mouse.

2.9. Cell tracking analysis

For tracking of ECFCs into ischemic tissues, the effects of recombinant periostin proteins on homing of intravenously transplanted ECFCs into the ischemic limbs of BALB/CA-nu/nu athymic nude mice (age 8–10 wks and weighing 17–22 g; eight mice per each experimental group) were determined. ECFCs were labeled with the long-lasting cell tracker CM-DiI according to the manufacturer's instructions. CM-DiI-labeled ECFCs (1×10^6 cells) were transplanted into the hindlimb ischemia animal model by intravenous injection into the tail vein, followed by direct injection of recombinant periostin proteins or PBS (60 μl each) into the ischemic limbs. For quantitative analyses, three sections measuring 6 μm in thickness were taken from the specimens at 150 μm intervals. CM-DiI-labeled cells were counted in three randomly selected microscopic fields from serial sections in each tissue block.

2.10. Statistical analysis

Results of multiple observations are presented as mean ± SD. For multivariate data analysis, one-way or two-way ANOVA, followed by Scheffé's post hoc test, was used for assessment of group differences.

3. Results

3.1. Periostin promotes migration of ECFCs through the first FAS-1 domain

To identify the functional domains involved in periostin-stimulated angiogenesis, we generated five fragments of periostin (Fig. 1A). The recombinant proteins were over-expressed as His-tagged proteins in *Escherichia coli* and purified using Ni-NTA affinity chromatography, and the purity of the purified periostin domains was estimated to be approximately 90% (Fig. 1B).

To determine whether periostin has pro-angiogenic potential, we performed a transwell migration assay in order to examine the effects of the recombinant full length periostin and its five fragments on the migration of ECFCs. As shown in Fig. 1C, the full length periostin stimulated migration of ECFCs as potent as VEGF, which was previously reported as a chemotactic factor for ECFCs [8]. We found that the first FAS-1 domain of periostin, but not other domains, induced significant stimulation of the chemotactic migration ability of ECFCs, and that the stimulatory effect of the first FAS-1 domain of periostin was as potent as that of the full length periostin protein (Fig. 1C). In addition, the first FAS-1 domain of periostin induced dose-dependent augmentation of ECFC migration with maximal stimulation at 10 μg/ml, and then declined at a concentration of 25 μg/ml (Fig. 1D).

3.2. The first FAS-1 domain of periostin stimulates adhesion of ECFCs

Because periostin is an extracellular adhesion molecule, we next explored the effect of periostin on the adhesive capacity of ECFCs. Precoating of 96-well culture plates with recombinant periostin resulted in enhanced adhesion of ECFCs onto culture plates. In addition, coating with the first FAS-1 domain of periostin, but not other periostin domains, resulted in significant enhancement of the adhesive capacity of ECFCs (Fig. 2A). In addition, the first FAS-1 domain of periostin induced a dose-dependent increase in the adhesive capacity of ECFCs with a maximal stimulation at a concentration of 10 μg/ml (Fig. 2B). These results suggest that the first FAS-1 domain of periostin is responsible for periostin-stimulated adhesion of ECFCs.

3.3. The first FAS-1 domain of periostin stimulates endothelial tube formation of human ECFCs in vitro

To investigate the angiogenic activities of the first FAS-1 domain of periostin, the effects of the full length and the five domains of periostin on tube forming ability of ECFCs were determined. Treatment of ECFCs with the full length periostin protein stimulated capillary-like tube formation in ECFCs. In addition, the first FAS-1 domain, but not other fragments of periostin, induced significant stimulation of tube forming ability of ECFCs (Fig. 3A and B). Endothelial tube formation induced by the first FAS-1 domain of periostin was maximally stimulated by treatment of ECFCs with a 2 μg/ml concentration (Fig. 3C), while migration and adhesion of ECFCs were maximally stimulated by treatment with 10 μg/ml of the first FAS-1 domain of periostin. Endothelial tube formation of ECFCs was significantly induced in response to treatment with the full length or the first FAS-1 domain of periostin as it was with VEGF treatment. Endothelial tube formation and maturation were observed over 9–16 h, and the capillary-like structure was maintained over 24 h (Supplemental Fig. 1).

3.4. Role of integrins β3 and β5 in the periostin-induced migration of ECFCs

Periostin has been reported to activate various cellular responses by activation of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [20]. To explore the involvement of integrins in periostin-stimulated cellular responses, ECFCs were pre-incubated with function-blocking antibodies against β1, β3, and β5 integrins, followed by measurement of cell migration in response to the full length and the first FAS-1 domain of periostin. Pre-incubation of ECFCs with antibodies against integrin β3 or β5 resulted in markedly attenuated cell migration stimulated by the full length or the first FAS-1 domain of periostin; however, incubation with anti-β1 antibody did not result in the abrogation of periostin-stimulated cell migration (Fig. 4). This result suggests involvement of β3 and β5 integrins in ECFC migration induced by the full length or the first FAS-1 domain of periostin.

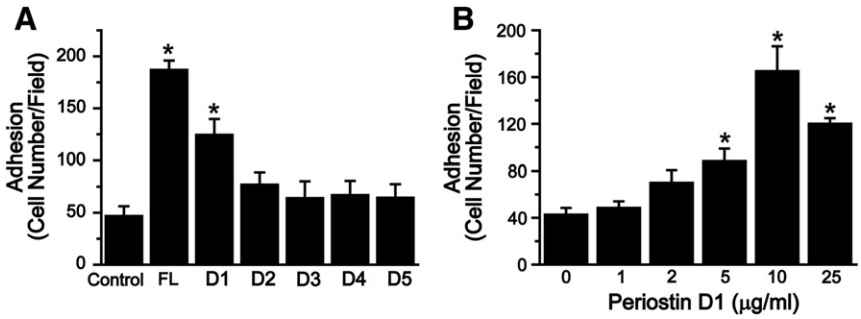

Fig. 2. Effects of the full length and the first FAS-1 domain of periostin on the adhesion of ECFCs. (A) Stimulation of ECFC adhesion by the first FAS-1 domain of periostin. 96-Well culture plates were coated with the full length periostin (FL) or the five fragments of periostin (10 μg/ml), followed by the determination of ECFC adhesion. (B) Dose-dependent effects of the first FAS-1 domain of periostin (periostin D1) on adhesion of ECFCs. Data represent mean ± S.D. (n = 4). * indicates $p < 0.05$ *vs* control.

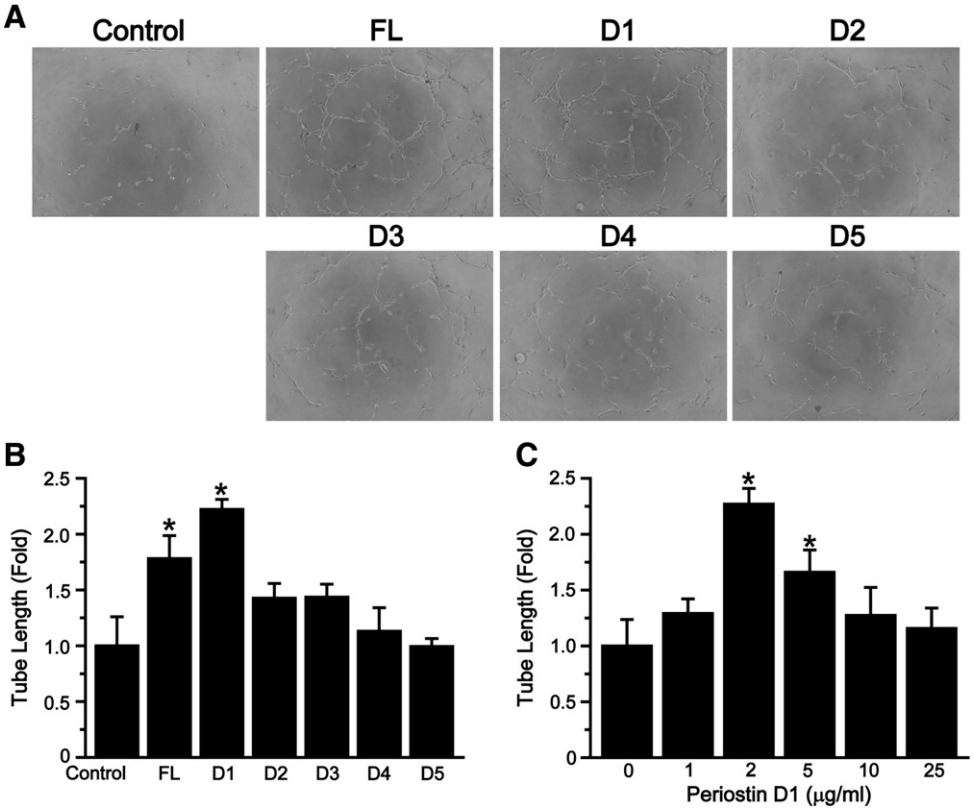

Fig. 3. Effects of the full length and the first FAS-1 domain of periostin on the tube formation of ECFCs. (A) Representative images of the tube formation of ECFCs in response to the full length (FL) or the five fragments of periostin (each 10 μg/ml). (B) Tube formation was quantified by measuring the length of tubes in four random fields from each well and normalizing the values relative to those of the corresponding control. (C) Dose-dependence of periostin D1 on tube forming ability of ECFCs. Data represent mean ± S.D. (n = 8). * indicates $p < 0.05$ *vs* control.

3.5. Intramuscular injection of periostin stimulates blood perfusion of the ischemic hindlimb and inhibits ischemic tissue damage

Because the full length and the first FAS-1 domain of periostin enhanced migration, adhesion, and tube formation of ECFCs *in vitro*, we next examined the effects of the full length and the first FAS-1 domain of periostin on *in vivo* angiogenesis and ischemic tissue damage in a murine model of hindlimb ischemia. The full length or the first FAS-1 domain of periostin was injected intramuscularly into the ischemic hindlimb, and blood flow was measured over a period of four weeks using an LDPI analyzer. Intramuscular injection of the full length or the first FAS-1 domain of periostin into the ischemic hindlimb resulted in significantly improved blood perfusion, as determined by LDPI (Fig. 5A and B). In addition, injection of the full length or the first FAS-1 domain of periostin resulted in significantly attenuated tissue necrosis and amputation as compared with control groups at four weeks after induction of ischemia (Fig. 5C).

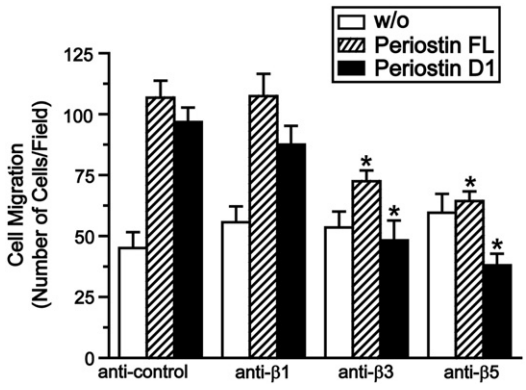

Fig. 4. Effects of function-blocking antibodies against integrins β1, β3, and β5 on ECFC migration. hECFCs were pre-incubated with control antibody or function-blocking antibodies against integrins β1, β3, or β5, followed by the determination of cell migration in response to the full length (periostin FL) periostin or the first FAS-1 domain of periostin (periostin D1) (each 10 μg/ml). Data represent mean ± S.D. (n = 6). * indicates $p < 0.05$ *vs* control.

To determine whether injection of the full length or the first FAS-1 domain of periostin can stimulate angiogenesis *in vivo*, we performed immunostaining for determination of densities of CD31-positive capillaries and α-SMA-positive arteries/arterioles in ischemic muscles. Intramuscular injection of recombinant periostin protein time-dependently increased the numbers of CD31-positive capillaries and α-SMA-positive blood vessels compared with control group in the ischemic hindlimb (Supplemental Fig. 2A–D). The density of CD31-positive capillaries was significantly higher in the ischemic limb injected with the full length or the first FAS-1 domain of periostin than that of the control groups injected with saline buffer (Fig. 6A and B). In addition, the numbers of α-SMA-positive blood vessels in the ischemic limb injected with the full length or the first FAS-1 domain of periostin were greater than those of the control groups (Fig. 6A and C). The increased numbers of CD31-positive capillaries and α-SMA-positive arterioles/arteries after injection of the full length or the first FAS-1 domain of periostin correlate with the increased blood perfusion in the ischemic limb injected with the full length or the first FAS-1 domain of periostin. The ischemic hindlimbs exhibited massive myofiber damage, inflammatory cell infiltration, and tissue fibrosis; however, intramuscular injection of the full length or the first FAS-1 domain of periostin attenuated the necrosis and fibrosis of the ischemic limbs (Supplemental Fig. 2E and A). Moreover, infiltration of $CD68^+$ monocytes/macrophages was inhibited in the ischemic limbs by injection of the full length or the first FAS-1 domain of periostin (Supplemental Fig. 3B and C). Injection of recombinant periostin proteins did not affect muscle architecture and fibrosis status in the normal hindlimbs (Supplemental Fig. 3D). These results

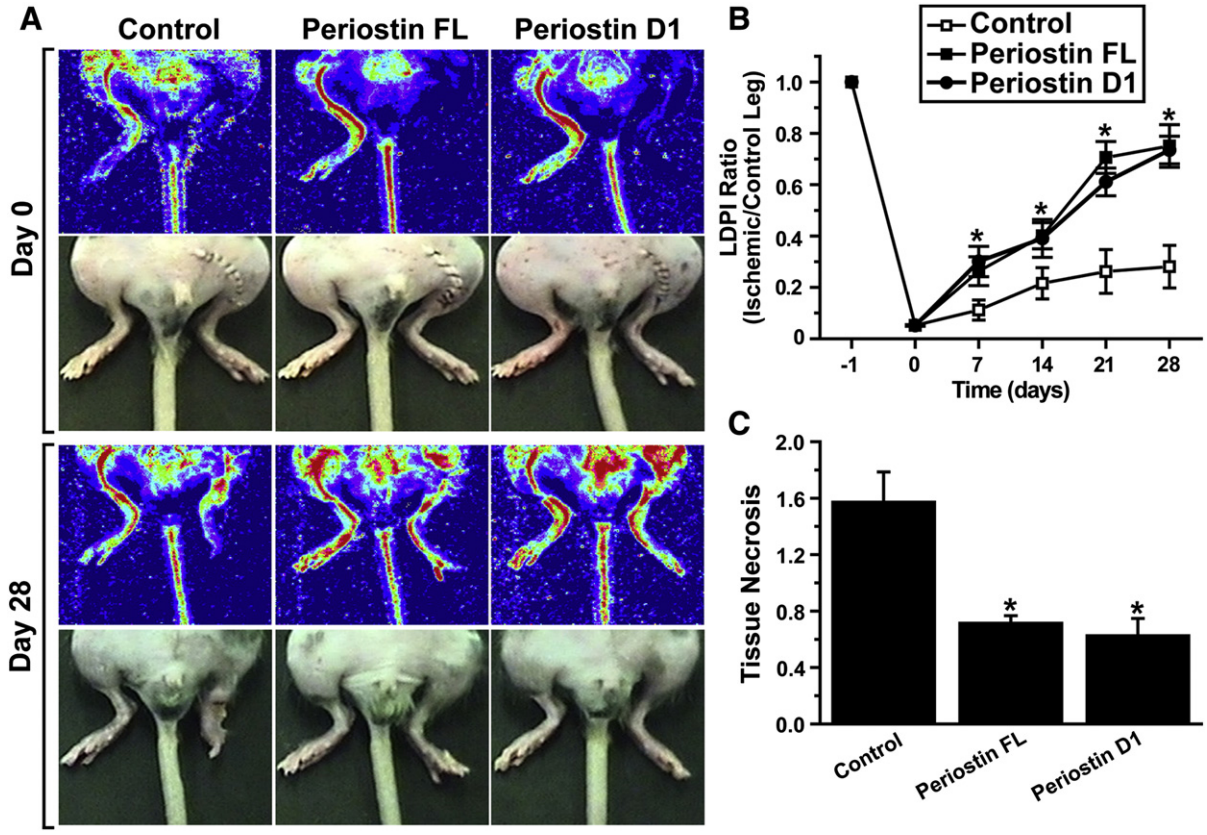

Fig. 5. Effects of the full length and the first FAS-1 domain of periostin on blood perfusion and limb salvage in an ischemic hindlimb animal model. The full length (periostin FL) or the first FAS-1 domain (periostin D1) of periostin (60 μg/each limb) was administered by intramuscular injection into the ischemic hindlimbs three times per week for a period of four weeks. (A) Representative images of the hindlimbs and blood flow measured by LDPI on days 0 and 28 from eight different mice. (B) Quantitative analysis of blood flow measured by LDPI. The LDPI ratio was calculated as the ratio of ischemic to contralateral hindlimb blood perfusion over the observation period. Data represent mean ± S.D. (n = 8 per each group). * indicates $p < 0.05$ periostin FL and D1 *vs* control. (C) Statistical analysis of the necrosis score on day 28. Data represent mean ± S.D. (n = 8 per group). * indicates $p < 0.05$ *vs* control.

suggest that intramuscular injection of the full length or the first FAS-1 domain of periostin provides protection from tissue necrosis and excessive inflammation in the ischemic hindlimb by stimulating blood perfusion.

3.6. Periostin stimulates homing and engraftment of ECFCs into the ischemic limb

To determine whether periostin can stimulate homing and engraftment of ECFCs *in vivo*, we examined the effects of the full length and the first FAS-1 domain of periostin on homing and engraftment of ECFCs into the ischemic limb *in vivo*. To trace the engraftment of ECFCs into the ischemic limb, ECFCs were labeled with the fluorescent dye CM-DiI, followed by i.v. injection of CM-DiI-labeled ECFCs and intramuscular injection of the full length or the first FAS-1 domain of periostin into the ischemic limbs. Sections of the ischemic hindlimb muscle were visualized under a confocal microscope, and engraftment of administered ECFCs was quantified on day 3 after i.v. injection of CM-DiI-labeled ECFCs. As shown in Fig. 7A, CM-DiI-positive cells were overlaid with CD31-positive cells in the ischemic limbs, suggesting incorporation of transplanted ECFCs into CD31-positive capillaries. The numbers of CM-DiI- and CD31-positive endothelial cells in the hindlimbs injected with the full length or the first FAS-1 domain of periostin were significantly higher than those in the control hindlimbs (Fig. 7A). In addition, engraftment of CM-DiI-labeled cells into the ischemic limbs was stimulated by intramuscular injection of the full length or the first FAS-1 domain of periostin (Fig. 7A and B). These results suggest that periostin promotes homing and engraftment of ECFCs into the ischemic limbs through the first FAS-1 domain-dependent mechanism.

4. Discussion

In the current study, we demonstrated that periostin stimulated angiogenic capacity of ECFCs, such as migration, adhesion, and tube formation through the first FAS-1 domain-dependent mechanism. Periostin has been known to stimulate angiogenic activity of endothelial cells [20,23]. Periostin plays a key role in the pathogenesis and progression of cardiac valve diseases by controlling angiogenesis [24]. In addition, periostin stimulated proliferation, migration, and lymphangiogenesis of lymphatic endothelial cells [25]. Moreover, periostin increased the sensitivity of endothelial cells to vascular endothelial growth factor through induction of VEGFR-2 expression [20]. We found that the full length and the first FAS-1 domain of periostin stimulated the endothelial tube formation of ECFCs and human umbilical vein endothelial cells (Supplemental Fig. 4). Therefore, it is likely that both ECFCs and tissue-resident endothelial cells are implicated in the periostin-stimulated vasculogenesis and angiogenesis in the ischemic limb. Although engraftment of administered ECFCs into the ischemic limbs was markedly stimulated by intramuscular injection of recombinant periostin, the percentages of CM-DiI-positive ECFCs incorporated into newly formed capillaries were quite low in the ischemic limb. The absolute number of incorporated ECFCs has been reported to drastically vary between 0% and 90% in the different studies [26]. Therefore, it is still unclear whether newly formed capillaries are originated from ECFCs, and the origin of endothelial cells in capillaries needs to be clarified further.

The FAS-1 domain of βig-h3 (TGF-β-induced protein) has been reported to inhibit vascular endothelial growth factor-induced growth and migration of endothelial cells by inhibiting the association of αvβ3 integrin with vascular endothelial growth factor receptor-2 [27]. Five fragments of periostin including the first FAS-1 domain, however,

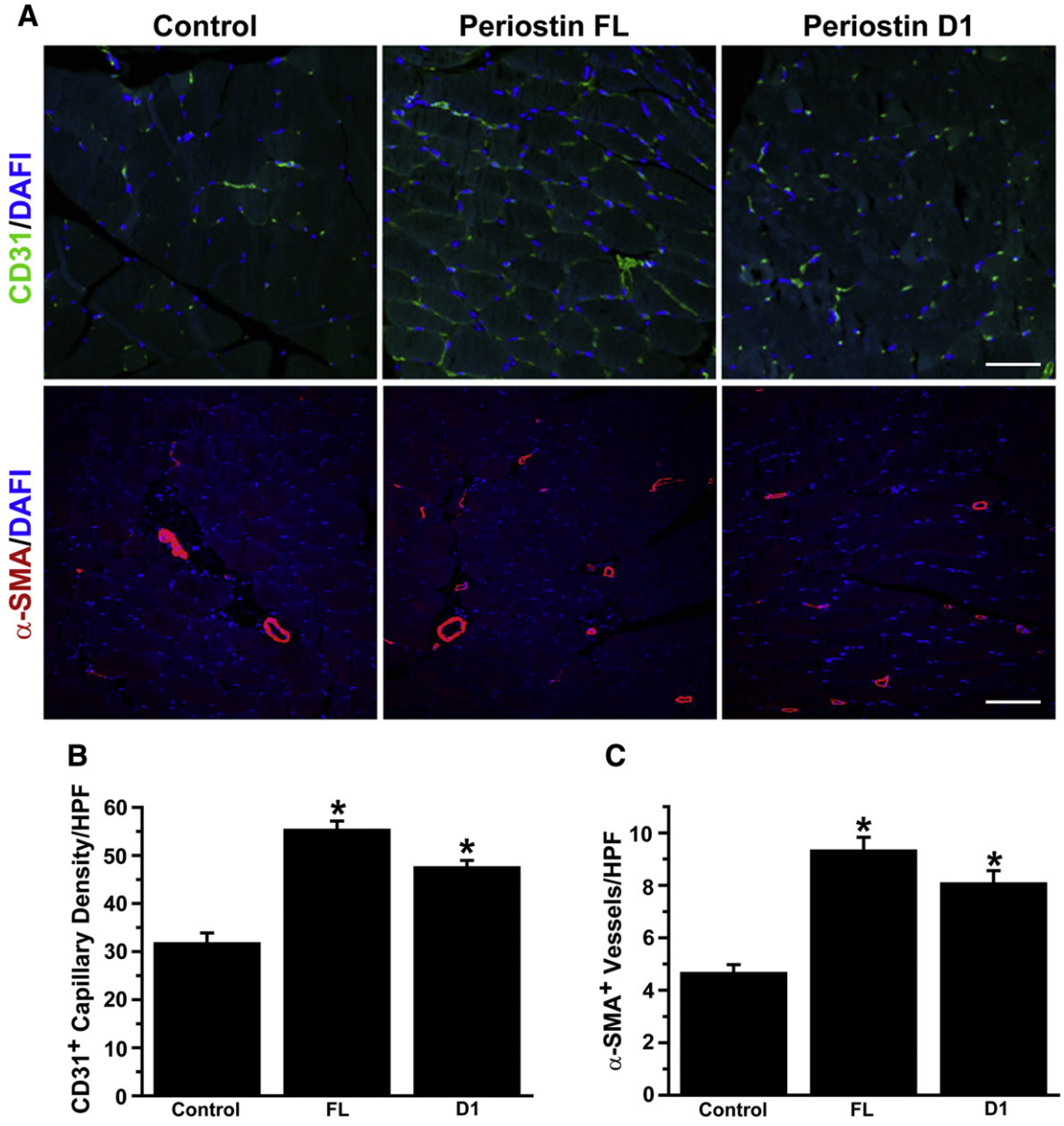

Fig. 6. Effects of the full length and the first FAS-1 domain of periostin on neovascularization in the ischemic hindlimb. (A) Immunostaining of CD31-positive capillaries (green) or α-SMA-positive blood vessels (red) in the ischemic limbs on day 28 after intramuscular injection of PBS (control), periostin FL, or periostin D1. Nuclei (blue) were counterstained with DAPI, and overlaid images are shown. Scale bar = 50 μm. (B) Quantitative analysis of capillary density expressed by the number of CD31-positive capillaries per high power field (HPF). (C) Quantitative analysis of α-SMA-positive vessels per HPF. Data represent mean ± S.D. (n = 24). * indicates $p < 0.05$ *vs* control.

did not affect the migration of ECFCs induced by the full length periostin (Supplemental Fig. 5), which suggests that each domain of periostin does not act as a dominant negative for the full length periostin. Function-blocking antibodies against integrin β3 and β5 subunits abrogated the cell migration stimulated by the full length or the first FAS-1 domain of periostin. It has been suggested that the FAS-1 domain of periostin is responsible for the direct interaction of periostin with several integrins, including $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [16]. Periostin promoted the adhesion and migration of ovarian epithelial cells through the activation of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [14]. Periostin mediated the migration of vascular smooth muscle cells through the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and focal adhesion kinase pathway [28]. In addition, $\alpha_v\beta_3$ integrin mediated the periostin-stimulated expression of MMP-2 [29]. DNA aptamer directed against human periostin caused disruption of the interaction between periostin and its cell surface receptors, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins [30]. The anti-periostin aptamer induced marked attenuation of the periostin-induced adhesion, migration, and invasion of breast cancer cells by blocking the activation of integrin receptor-mediated signaling. These results support those of the current study, indicating that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ play a critical role in the stimulation of angiogenic activities of ECFCs by direct interaction with periostin.

In this study, we demonstrated that the full length or the first FAS-1 domain of periostin significantly improved blood perfusion and attenuated tissue necrosis in the ischemic limbs, compared with the control group. In addition, the numbers of CD31-positive capillaries and α-SMA-positive arteries were increased in the ischemic limbs injected with the full length or the first FAS-1 domain of periostin. Recombinant periostin protein has been reported to stimulate the proliferation of cardiomyocytes *in vitro* and promoted cardiac repair and angiogenesis after myocardial infarction [31], whereas periostin knockout mice exhibited impaired cardiac healing after acute myocardial infarction [32]. Periostin-deficient mice exhibited delayed *in vivo* wound repair, and direct administration of exogenous periostin resulted in improved wound repair [33]. Periostin-overexpressing mesenchymal stem cells improved cardiac function compared to mesenchymal stem cell-injected controls, when they were injected into the infarcted myocardium [34]. These

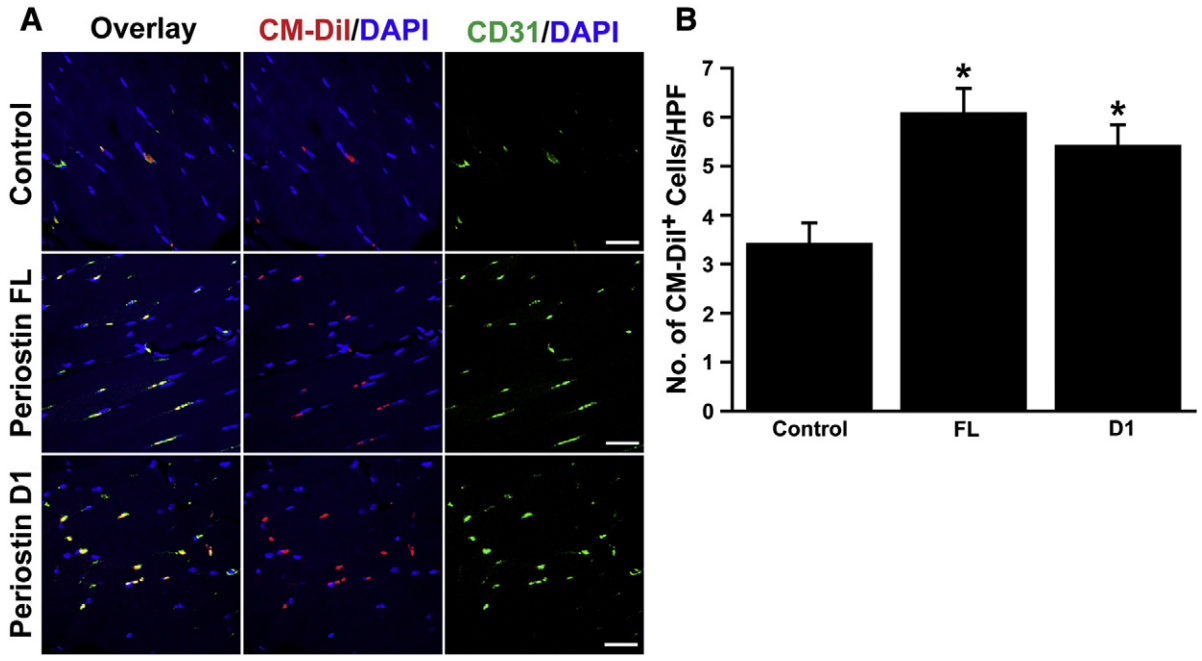

Fig. 7. Effects of the full length and the first FAS-1 domain of periostin on the engraftment of ECFCs. (A) ECFCs were labeled with CM-DiI and injected intravenously with intramuscular injection of PBS (control), periostin FL, or periostin D1. Tissue sections of the ischemic hindlimb of day 3 after ECFC injection were immunostained with anti-CD31 antibody. Overlaid images of CM-DiI-positive ECFCs (red color), nuclei (blue color), and CD31-positive endothelial cells (green color) are shown. (B) Quantitative analysis of the number of CM-DiI-labeled ECFCs per HPF. Data represent mean ± S.D. (n = 24). * indicates $p < 0.05$ *vs* control.

results suggest a pivotal role of periostin in the repair of cardiovascular tissues. Intrapericardial delivering of recombinant peptide consisting of the four FAS-1 domain of human periostin stimulated myocardial repair but increased myocardial fibrosis in a pig model of myocardial infarction [35]. Furthermore, periostin has been reported to promote fibrosis in polycystic kidney disease and in an animal model of idiopathic pulmonary fibrosis [36,37]. In the present study, we showed that the intramuscular injection of recombinant periostin attenuated tissue fibrosis and excessive inflammation in the ischemic limbs. The molecular mechanism of reduced fibrosis in the ischemic limbs injected with recombinant periostin proteins is still unclear. While macrophages are beneficial to the repair of normally healing wounds, they may promote excessive inflammation and fibrosis in certain circumstances [38]. Therefore, it is likely that increased blood perfusion can prevent tissue necrosis and excessive inflammation which may lead to tissue fibrosis.

Periostin has been known to be expressed in response to tissue injury, including myocardial infarction and cutaneous wound [39]. It has been reported that periostin expression was strongly up-regulated in the granulation tissues of the wounded skin, whereas it could be detected at the low level in the dermal-epidermal junction and around the hair follicles in the normal unwounded skin [33]. Consistently, we found that periostin expression was induced in the granulation tissues of the wounded skin (Supplemental Fig. 6). In contrast, we could not detect the expression of periostin in the muscle tissues of the normal hindlimbs and ischemic hindlimbs. These results suggest that periostin expression can be induced in the wounded skin but not in the ischemic limbs. Therefore, it is likely that the therapeutic effect of recombinant periostin protein does not simply accentuate a naturally-occurring process but stimulate the regenerative function of ischemic tissues.

Intramuscular injection of the full length or the first FAS-1 domain of periostin stimulated homing of intravenously transplanted human ECFCs into the ischemic limbs and incorporation of ECFCs into newly formed $CD31^+$ capillaries. Various angiogenic factors, such as VEGF, SDF-1α, and insulin-like growth factor2 (IGF2), all of which are up-regulated in hypoxic condition, have been reported to regulate recruitment of ECFCs [40]. Even though the intra-arterial injection of VEGF promotes revascularization in a rabbit ischemic hindlimb model [41], local delivery of VEGF-A may increase the risk of plaque destabilization in advanced atherosclerosis [42]. In myocardial infarction, up-regulation of SDF-1α occurs immediately after the introduction of infarction; however, it is down-regulated within seven days [43]. According to the results of our study, periostin promoted the adhesion of ECFCs through the activation of β_3 or β_5 integrins. Integrins represent a major molecular determinant of ECFC function, with different integrin subunits regulating different steps of ECFC biology. Specifically, integrin $\alpha_4\beta_1$ is a key regulator of ECFC retention and/or mobilization from the bone marrow, while integrins $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ are major determinants of ECFC homing, invasion, differentiation, and paracrine factor secretion [44]. Integrin $\alpha_v\beta_3$ has been reported to play a key role in angiogenesis by regulating migration and adhesion of ECFCs in an ischemic hindlimb animal model [45]. In addition, integrin-mediated adhesion to the extracellular matrix is essentially required for the incorporation of circulating ECFCs into the blood vessels and differentiation into the endothelial cells [46]. Specifically, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins play a pivotal role in ECFC adhesion to denuded vessels and re-endothelialization of denuded arteries [47]. These results support those of the current study showing that periostin-induced activation of integrins plays a critical role in the neovascularization of ischemic tissues by eliciting migration, adhesion, and tube formation of ECFCs.

Numerous clinical trials evaluating the therapeutic effects of circulating ECFCs are currently in progress [48]. However, most patients undergoing ECFC therapy for ischemic diseases have background problems hindering the efficacy of autologous ECFC therapy. Therefore, finding extracellular factors to enhance the recruitment of ECFCs into ischemic tissues brings scientific understanding of ECFC biology as well as providing a tool for treatment of ischemic diseases. Compared to the full length periostin, the first FAS-1 domain of periostin could be highly expressed in *E. coli* and large amounts of the FAS-1 domain peptide of periostin could be efficiently purified to homogeneity. Therefore, the first FAS-1 domain peptide can be used for the enhancement of therapeutic efficacy of ECFCs in treatment of ischemic diseases, such as peripheral arterial diseases, myocardial infarction, and stroke, by stimulating the therapeutic potential of endogenous or transplanted ECFCs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.05.004.

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